ZooKeys 1257: 305–326 (2025)
DOI: 10.3897/zookeys.1257.163020

Research Article

First DNA barcodes and morphometric analysis of Singaporean Tetrigidae (Orthoptera, Caelifera) support color variations as intraspecific polymorphism through integrative taxonomy

Ivan Neo[1], Rebecca Ker Loh[1], Tricia J. Y. Cho[2], Nalini Puniamoorthy[1], Darren C. J. Yeo[1,2], Ming Kai Tan[2]

1 *Department of Biological Sciences, National University of Singapore, 16 Science Drive 4, Singapore 117558, Singapore*

2 *Lee Kong Chian Natural History Museum, Faculty of Science, National University of Singapore, 2 Conservatory Drive, Singapore 117377, Singapore*

Corresponding author: Ming Kai Tan (orthoptera.mingkai@gmail.com)

Abstract

Integrative taxonomy refers to the practice of incorporating multiple lines of evidence, including morphology and genetics, to delineate species. While both morphology and genetic markers, such as the *Cytochrome c oxidase subunit I* (*COI*), have been widely applied in insect taxonomy, their use remains limited in the taxonomic studies of pygmy grasshoppers (Orthoptera: Tetrigidae). Currently, DNA barcodes for tetrigids, especially from Southeast Asia, remain scarce. This limitation is especially pressing given the extensive color variation documented in pygmy grasshoppers, which has rarely been systematically tested to determine whether such variation represents intraspecific polymorphisms or species differences. Historically, color polymorphism has even led to erroneous species delimitation. In this study, we examined taxa from Singapore previously reported to exhibit color variation and applied an integrative taxonomic framework to investigate whether these morphs represent intraspecific polymorphism or species-level differences. Our analyses combined morphometrics of 22 characters with principal component analyses, and DNA barcoding using short gene fragments of *COI* and small *ribosomal RNA subunit III* (*12S*). We focused on four taxa: two morphospecies of *Coptotettix*, *Loxilobus insidiosus* (Bolívar, 1887), and *Thoradonta nodulosa* (Stål, 1861). DNA barcoding revealed that color morphs are genetically similar, and morphometric analysis likewise showed no distinct clustering. Together, these results indicate that different color morphs indeed represent intraspecific polymorphisms. More importantly, we demonstrate that combining molecular data with morphological evidence offers an effective, integrative framework for resolving taxonomic challenges within tetrigids and highlights the value of integrative approaches for clarifying species boundaries in morphologically variable taxa.

Key words: *COI*, molecular analysis, objective clustering, phylogenetics, pygmy grasshoppers, *12S*

OPEN ACCESS

Academic editor: Jun-Jie Gu
Received: 24 June 2025
Accepted: 27 September 2025
Published: 30 October 2025

ZooBank: https://zoobank.org/B3D4F2F0-A13D-4D8F-9C87-5201383956CB

Citation: Neo I, Loh RK, Cho TJY, Puniamoorthy N, Yeo DCJ, Tan MK (2025) First DNA barcodes and morphometric analysis of Singaporean Tetrigidae (Orthoptera, Caelifera) support color variations as intraspecific polymorphism through integrative taxonomy. ZooKeys 1257: 305–326. https://doi.org/10.3897/zookeys.1257.163020

Copyright: © Ivan Neo et al.
This is an open access article distributed under terms of the Creative Commons Attribution License (Attribution 4.0 International – CC BY 4.0).

Introduction

Color polymorphism is the existence of two or more distinctly colored phenotypes within an interbreeding population (Huxley 1955). This phenomenon is ubiquitous in animals (McKinnon and Pierotti 2010; Wellenreuther et al. 2014) and can be subject to both natural and sexual selection, including predator–prey interactions and mate preferences (e.g., Roulin and Wink 2004; Roulin and Bize 2007; Sánchez-Guillén et al. 2013). Investigation of its genetic basis and phenotypic interactions with the environment has offered us insights into evolution, including the mechanisms that maintain such polymorphism and its role in speciation (Hugall and Stuart-Fox 2012). In systematics, color polymorphism can correlate with phylogenetic relatedness and/or relate to geographical and environmental variations (Drosopoulos 2003; Messmer et al. 2005; Beukema et al. 2016). Consequently, color variations among insect taxa have always intrigued systematists and taxonomists because they are keen to ascertain whether such variations represent intraspecific polymorphism or distinct species differences (Honek and Furlan 1995; Nie et al. 2012; Blow et al. 2021; Ramaiah et al. 2023). Polymorphism can also complicate or lead to erroneous species delimitation. This has led to integrative approaches incorporating life history, morphology and phylogeny being adopted to address this question, in various insect groups including coleopterans, hemipterans, and odonates (e.g., Nie et al. 2012; Blow et al. 2021; Ramaiah et al. 2023), as well as orthopterans (Cueva Del Castillo et al. 2021; Fianco 2023).

Understanding of color variability remains limited, however, in numerous insect taxa. Among these are the pygmy grasshoppers (Orthoptera: Tetrigidae), a monophyletic clade representing the most primitive family within the suborder Caelifera (Song et al. 2015). Color polymorphism is extensive among tetrigids (Nabours 1929; Nabours and Foster 1929; Holst 1986; Ichikawa et al. 2006; Zhao et al. 2016), including species from Southeast Asia (Forsman 2000; Adžić, 2021; Neo et al. 2023). Among tetrigid species known to display color polymorphism, such as *Tetrix japonica* (Kaori et al. 2010), *Tetrix undulata* (Ahnesjö and Forsman 2003), and *Tetrix subulata* (Tetriginae: Tetrigini) (Forsman and Appelqvist 1998; Forsman 1997, 2000), polymorphism in color and patterns can be discrete and/or continuous.

Despite the widespread reports of color polymorphism in tetrigids, few studies have rigorously tested species delimitation of these color polymorphic taxa using an integrative taxonomic approach. Integrative taxonomy refers to the practice of incorporating multiple lines of evidence, including morphology and genetics, to delineate species in taxonomy. There is a pressing need to continue apply integrative taxonomic approaches to tetrigids, as they have been shown to effectively distinguish intraspecific color and pattern variations from species-specific differences in the family (Zhao et al. 2016). Historically, color morphs have often been mistaken to be distinct species (e.g., Bolívar 1887; Hancock 1907; Hancock 1909; Günther 1939; Devriese 1996; Long et al. 2023). This misidentification largely arose because, unlike other orthopterans, tetrigids lack reliable diagnostic morphological characters (Tumbrinck 2014; Tumbrinck and Skejo 2017; Muhammad et al. 2018; Zha et al. 2021; Skejo et al. 2022). Specifically, the male genitalia, which are often sclerotized and bear many reliable taxonomic characters used for diagnosing orthopteran species (Song 2010; Chamorro-Rengifo and Lopes-Andrade 2014; Gorochov 2015),

typically do not offer diagnostic features for tetrigids. This calls for the use of a more integrative approach, considering additional lines of evidence, such as molecular or morphometric data.

In integrative taxonomy, morphometric and genetic data are frequently used as complementary lines of evidence to attain a more holistic understanding of species delimitations in the taxa of interest. Morphometric analysis provides a more quantitative approach for examining morphology and has recently emerged as a rigorous approach for delimiting species within tetrigids (Tumbrinck 2014; Tan et al. 2017; Pan et al. 2018; Tan et al. 2019; Moser et al. 2021). This method is particularly useful for cryptic species exhibiting subtle morphological differences that are difficult to discern visually without quantitatively measuring and comparing characters (Tan et al. 2017). Traditional morphometric approaches involve measuring body part lengths and comparing their ratios (Pan et al. 2018; Moser et al. 2021), whereas more recent methods employ distance-based cluster analyses such as Principal Component Analysis (PCA) and neighbor-joining (NJ) trees. These analyses identify possible diagnostic characters influenced by speciation, and work on the assumption that all body measurements are interrelated (Tan et al. 2017; Pan et al. 2018; Tan et al. 2019; Moser et al. 2021).

The application of genetic data to objectively test species delimitation hypotheses in tetrigids has emerged recently (e.g., Zhao et al. 2016; Tan et al. 2017; Kasalo et al. 2025; Fite et al. 2025). DNA barcodes, such as the *cytochrome c oxidase subunit I* (*COI*), have been used to construct gene trees to infer species delimitations (Kasalo et al. 2023, 2025; Fite et al. 2025). These above-mentioned studies, however, have largely excluded color-variable taxa. Integrating DNA barcode information could greatly enhance tetrigid species delimitation, especially since coalescent-based methods have statistical rigor and offer an additional approach to determine whether color morphs observed represent within-species color polymorphisms or distinct species.

In recent surveys, we observed color variations in four putative species identified as *Coptotettix* morphospecies 1, *Coptotettix* morphospecies 2, *Loxilobus insidiosus* (Bolívar, 1887) (Criotettiginae: Thoradontini), and *Thoradonta nodulosa* (Stål, 1861) (Criotettiginae: Thoradontini) (see Neo et al. 2023) (Fig. 1) collected from the Nee Soon freshwater swamp forest in Singapore (Neo et al. 2023). Neo et al. (2023) postulated that these color morphs are probably intraspecific variants based on current taxonomic literature, but did not test this hypothesis via an integrative taxonomic approach. By employing both DNA barcodes and morphometry, we test whether the color variations in these taxa are indeed distinct species or represent interspecific color polymorphism. Putative species units were obtained using DNA barcodes (i.e., molecular operational taxonomic units or mOTUs) using two mitochondrial gene fragments: *COI* and *small ribosomal RNA subunit III* [*12S*]). Molecular species delimitation was done using an objective clustering method (following the principles of SpeciesIdentifier, see Meier et al. 2006), as well as a coalescent-based species delimitation method (i.e., Assemble Species by Automatic Partitioning analysis, see Puillandre et al. 2021). We also performed a morphometric distance-based cluster analysis using 22 characters. We are hitherto not aware of any study that uses such an integrative approach to address the color variability in tetrigids in Southeast Asia. By using color polymorphic taxa as a case study, we aim to show how integrative taxonomy can quickly help resolve taxonomic problems in tetrigids, including over-splitting of polymorphic taxa.

Materials and methods

Material examined and identification

Between November 2022 and February 2023, tetrigids were opportunistically collected from Nee Soon Swamp Forest (NSSF), Singapore (Suppl. material 1). Permission to conduct faunistic surveys and collection of material was granted by the National Parks Board (NP/RP22-096 and NP/RP22-113). Specimens were imaged for morphometric measurements and identification (see Neo et al. 2023), then stored in −80 °C freezer. The grasshoppers were identified as putative species, using photographs and descriptions of type specimens, comparison with other specimens in the Zoological Reference Collection (**ZRC**) of the Lee Kong Chian Natural History Museum (**LKCNHM**), as per Neo et al. (2023). When color variations were observed in the putative species, we sorted them into different color morphs:

For *Coptotettix* morphospecies 1 (Fig. 1A):

A: Uniformly dark pronotal coloration, pale femur stripe (when viewed laterally)
B: Dark pronotal coloration, pale lateral lobes
C: Uniformly pale pronotal coloration, banded femur (when viewed laterally)
D: Pale pronotal coloration, dark lateral lobes

For *Coptotettix* morphospecies 2 (Fig. 1B):

A: Uniformly dark pronotal coloration
B: Dark pronotal coloration, pale dorsal band across center of pronotum
C: Pale pronotal coloration, dark lateral lobes
D: Pale pronotal coloration, multiple dorsal stripes along center of pronotum

For *Loxilobus insidiosus* (Fig. 1C):

A: Dark pronotal coloration, pale lateral lobes
B: Uniformly pale pronotal coloration
C: Half pale (anterior) half dark (posterior) pronotal coloration

For *Thoradonta nodulosa* (Fig. 1D):

A: Uniformly dark pronotal coloration
B: Uniformly pale pronotal coloration
C: 3 pale pronotal patches (1 posterior to head, and 2 mid lateral of pronotum)
D: 2 pale pronotal patches (2 mid lateral of pronotum)
E: Half pale (anterior) half dark (posterior) pronotal coloration
F: 1 pale pronotal patch posterior to head
G: Pale pronotal coloration, paler band across pronotum
H: Dark pronotal coloration, pale lateral lobes

In our dataset, we also included two non-polymorphic but morphologically cryptic species, i.e., *Loxilobus neesoon* and *Loxilobus simulans* (Criotettiginae:

Thoradontini). These two species will serve as a control and test-bed for both the species delimitation and morphometric analysis. We expect that both analyses will reveal these as two distinct taxa.

Morphometric measurements

Measurements of 22 morphological characters were made for each specimen, whenever possible, as described in Neo et al. (2023) (Suppl. material 1). Each specimen was photographed and digitally measured using ImageJ 1.53t (Wayne Rasband, Research Services Branch, National Institute of Mental Health, Bethesda, MD, USA) (Neo et al. 2023: table 1). For the measurements, the abbreviations of the characters follow those in Neo et al. (2023):

PL Pronotal disc length
PW Pronotum disc width
PLW Pronotal lateral lobe width
VW Vertex width
EW Eye width
PAM Width of anterior margin of pronotal disc
PCW Prozonal carina width
PCL Prozonal carina length
PH Pronotum height
IAH Infra-scapular area height
TW Tegmen width
PTL Posterior tibia length
PFL Posterior femur length
PFW Posterior femur width
FHW Fastigial horn width
SW Scutellum width
CEH Clypeus-eye height
AGH Antennal groove-eye height
PML Pronotum anterior margin-notch length
NW Notch width
ICL Interhumeral carina length
CW Carina width

Morphometric analysis

To examine how color morphs may differ morphologically, measurements taken for putative species *Coptotettix* morphospecies 1, *Coptotettix* morphospecies 2, *Loxilobus insidiosus*, and *Thoradonta nodulosa* were summarized into major gradients of variation by performing PCA on scaled measurements using the 'pca.calc' function in the R package 'MorphoTools2' (Šlenker et al. 2022). Biplots were then observed for any clustering or driving traits differentiating the color morphs using the functions 'plotPoints' and 'plotAddSpiders'. We also tested whether PCA of the morphometric measurements can differentiate well-established cryptic species pair *Loxilobus simulans* and *Loxilobus neesoon*. Previously, Tan et al. (2017) also employed morphometry to separate these two cryptic species.

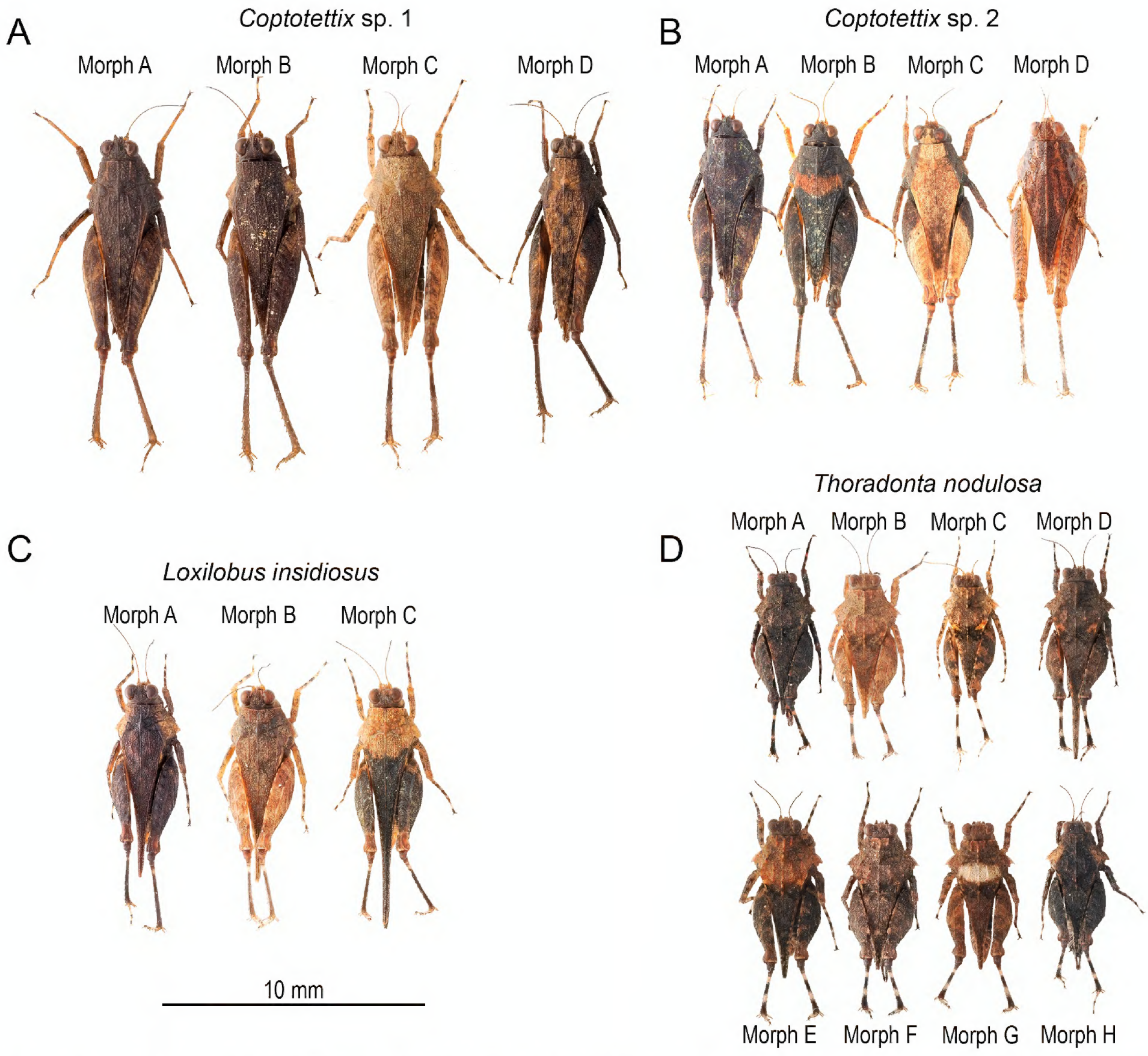

Figure 1. Dorsal view of the different color morphs. **A.** *Coptotettix* morphospecies 1; **B.** *Coptotettix* morphospecies 2; **C.** *Loxilobus insidiosus*; **D.** *Thoradonta nodulosa*.

Molecular protocols: DNA barcoding, sequencing, and bioinformatics

DNA extraction was done using the QuickExtract DNA Extraction Solution (ScientificResources), which allows for rapid DNA extraction by incubation alone. DNA template was obtained from specimens by plucking a leg, submerging it in QuickExtract solution, and incubating at 65 °C for 18 min, followed by 98 °C for 2 min.

PCR amplification was conducted using two sets of primers: universal metazoan *COI* primers mlCOIintF: 5'- GGWACWGGWTGAACWGTWTAYCCYCC -3' (Leray et al. 2013) and jgHCO2198: 5'- TAIACYTCIGGRTGICCRAARAAYCA -3' (Geller et al. 2013) which targeted and amplified the 313 bp *COI* mini-barcode, the *12S*F: 5'- TACTATGTTACGACTTAT -3' and *12S*R: 5'- AAACTAGGATTAGATACCC -3' (Chintauan-Marquier et al. 2016; Kambhampati, 1995). These primers were tagged with 13 bp indices, and possessed at least 4 base-pair differences and lacked homopolymers (Srivathsan et al. 2021), making them

suitable for MinION sequencing. Each PCR sample was assigned a unique combination of forward and reverse primer tags, which will facilitate downstream bioinformatic assignment to specimens. Negative controls, where DNA extract was replaced with molecular-grade water, were included for each batch of (at most) 95 PCR samples.

Regardless of the primer used, each PCR sample volume contained 8 µL of CWBio 2× Master Mix, 1 µL of 1 mg/mL BSA, 2 µL of 5 µM of each primer, and 3 µL of DNA extract (10× diluted). The thermocycling conditions varied for each gene fragment: for *COI*, a starting denaturation step of 5 min at 94 °C, followed by 40 cycles of 30 s at 94 °C, 45 s at 45 °C and 45 s at 72 °C, and a final extension step of 5 min at 72 °C; for *12S*, a step-up reaction starting with a denaturation step of 5 min at 94 °C, followed by 10 cycles of 30 s at 94 °C, 30s at 45 °C and 45 s at 72 °C, then 40 cycles of 30 s at 94 °C, 30 s at 47 °C and 45 s at 72 °C, and a final extension step of 5 min at 72 °C. After amplification, PCR products for the same gene fragment were pooled in equal volumes and purified using Ampure XP beads (Beckman Coulter, USA) as per manufacturer's instructions. The pools were then quantified using the dsBR kit on the Qubit fluorometer and equimolar combined.

Library preparation was conducted using the Ligation Sequencing Kit V12 (SQK-LSK112), and sequencing was done on the MinION mk1b. Although Kit V12 was used, we followed the library preparation protocol "Ligation Sequencing Amplicons V14", with one modification: during End-prep, DNA CS was replaced with molecular-grade water.

Raw FAST5 data was base-called using Guppy and the r10.3 super accurate model. The resulting FASTQ files were demultiplexed using ONTbarcoder (v. 0.1.9) (Srivathsan et al. 2021). Different quality check (QC) filters were applied to each gene fragment. For *COI*, the inbuilt filters of ONTbarcoder were applied, targeting protein-coding genes. Sequences with ambiguous bases were discarded, as we found that they would later interfere with pairwise-distance (p-distance) calculations for mOTU (molecular operative taxonomic units) clustering. The remaining sequences were uploaded to the GBIF's online search engine (https://www.gbif.org/tools/sequence-id) to get a preliminary taxonomic assignment. Only sequences matching ≥ 85% to tetrigids were retained, while others were treated as contaminated samples and discarded.

For *12S*, which is not a protein-coding gene, the inbuilt QC filters of ONTbarcoder could not be applied. Post-demultiplexing, sequences with ambiguous bases were discarded. The remaining sequences were queried against the GenBank and DNA barcode database using MegaBLAST from BLAST 2.13.0+ (Camacho et al. 2009) to attain a preliminary taxonomic assignment. Only sequences matching ≥ 85% to tetrigids were retained, while the rest were discarded.

For selected specimens, where DNA barcodes were not obtained, PCR amplification was repeated, and PCR products were sent to Macrogen Asia Pacific Private Limited for Sanger sequencing. The same PCR reagents and protocols described above were used, except that the PCR mastermix was replaced with the Vazyme 2× Rapid Taq Master Mix P222. Chromatographs of DNA sequences were viewed and trimmed using the default settings in Geneious Prime 2023.2.1 (Java Version 11.0.18). Sanger sequencing results revealed that for the following taxa and genes, the earlier blanket thresholds

we used for flagging contaminations (i.e., ≥ 85% match to tetrigids) was too stringent: *Coptotettix* morphospecies 1A, *Thoradonta nodulosa* morph E and *Potua coronata* for *COI*; *Loxilobus simulans* and *Potua coronata* for *12S*. These species and DNA barcodes sequences were absent from the existing online databases and did not match ≥ 85% to the existing tetrigid sequences available, even though it turned out that the DNA barcode sequence attained from both sequencing methods (MinION and Sanger sequencing) were exactly the same. For these taxa and genes, we made an exception and readed them into our dataset.

The sequences generated for this study were deposited in GenBank with accession numbers PP760259–PP760335 for *COI* and PP778511–PP778662 for *12S* (Suppl. materials 2, 3, respectively).

Species delimitation

To cluster specimens into putative species according to their DNA barcodes, we used an objective clustering program, which relies on pairwise-distances (p-distance), as well as the principles of SpeciesIdentifier (Meier et al. 2006). Objective clustering was performed for each gene (i.e., *COI* and *12S*) separately. A dendrogram was built for each gene, starting with the smallest pairwise distances. Subsequently, a coalescent-based species delimitation analysis known as the ASAP (Puillandre et al. 2021) was employed for each of the single locus sequence alignments, to determine the putative species units among tetrigid specimens. ASAP divides species partitions based on pairwise genetic distances, and computes a probability of panmixia (p-val), a relative gap width metric (W) and ranked results by the asap-score: the lower the score, the better the partitioning (Puillandre et al. 2021). Sequence alignments were done using MAFFT v. 7, through the web-server accessible at https://mafft.cbrc.jp/alignment/server/. The ASAP analysis was executed using the Kimura K80 substitution model (ts/tv = 2.0) through the web-server accessible at https://bioinfo.mnhn.fr/abi/public/asap.

Phylogenetic analysis

To conduct a preliminary examination of relationships between species, which cannot be accomplished using species delimitation analysis, we ran a phylogenetic analysis for *COI* with maximum likelihood (ML) using the using the iq-tree web server with the default options (http://iqtree.cibiv.univie.ac.at/; Trifinopoulos et al. 2016). We emphasize that this analysis is preliminary, as it relies primarily on short COI fragments, and should only be interpreted as a hypothesis to guide future, more comprehensive studies. We also combined our data with closely related or similar species (i.e., congenerics) from the region in which *COI* sequences are available on public depositories such as GenBank and BOLD. In total, 102 sequences were included in this preliminary phylogenetic analysis. We used ModelFinder to search for the best model among 88 available DNA substitution models. The best model was chosen according to Bayesian Information Criterion (BIC). Branch support was assessed by conducting 100 standard bootstrap replicates. Branches with bootstrap support values (BS) ≥ 75% were considered strongly supported.

Results

Species delimitation

Based on *COI* sequences of 76 specimens belonging to nine putative species, both the objective clustering analysis and ASAP revealed that *Coptotettix* morphospecies 1, *Coptotettix* morphospecies 2, *Loxilobus insidiosus*, and *Thoradonta nodulosa* can each be considered as a single putative species (Fig. 2, Suppl. material 4). For instance, in most insects, pairwise differences larger than 3% in the *COI* barcode are often used as a threshold to delineate separate species (Meier et al. 2016; Wang et al. 2018; Yeo et al. 2020). The lowest genetic distances between the nine putative species range from 11.25–19.61%, demonstrating that there is a clear gap in interspecific genetic distances. *Coptotettix* morphospecies 1, *Coptotettix* morphospecies 2 also differed by a p-distance of at least 12.62%. In contrast, the most genetically divergent specimen within morphospecies 1 had p-distance of as low as 0.64% to one other specimen in the cluster, and for *Loxilobus insidiosus* and *Thoradonta nodulosa* the p-distance is 0.32% and 0.32%, respectively. After a global-alignment, the applied ASAP procedure identified nine mOTUs (hypothetical species) at the threshold distance 7.35% which has the best asap-score (2.0), and a second partition with identical results and inference. DNA data for *Coptotettix* morphospecies 2 color morph C was available and was genetically similar to other color morphs, although no *COI* data was available for morph B.

Likewise, based on *12S* sequences of 152 specimens belonging to nine putative species, both the objective clustering analysis and ASAP revealed that color morphs observed in *Coptotettix* morphospecies 1, *Coptotettix* morphospecies 2, *Loxilobus insidiosus* and *Thoradonta nodulosa* are genetically similar, and can each be considered as a single species (Fig. 3, Suppl. material 5). Between the

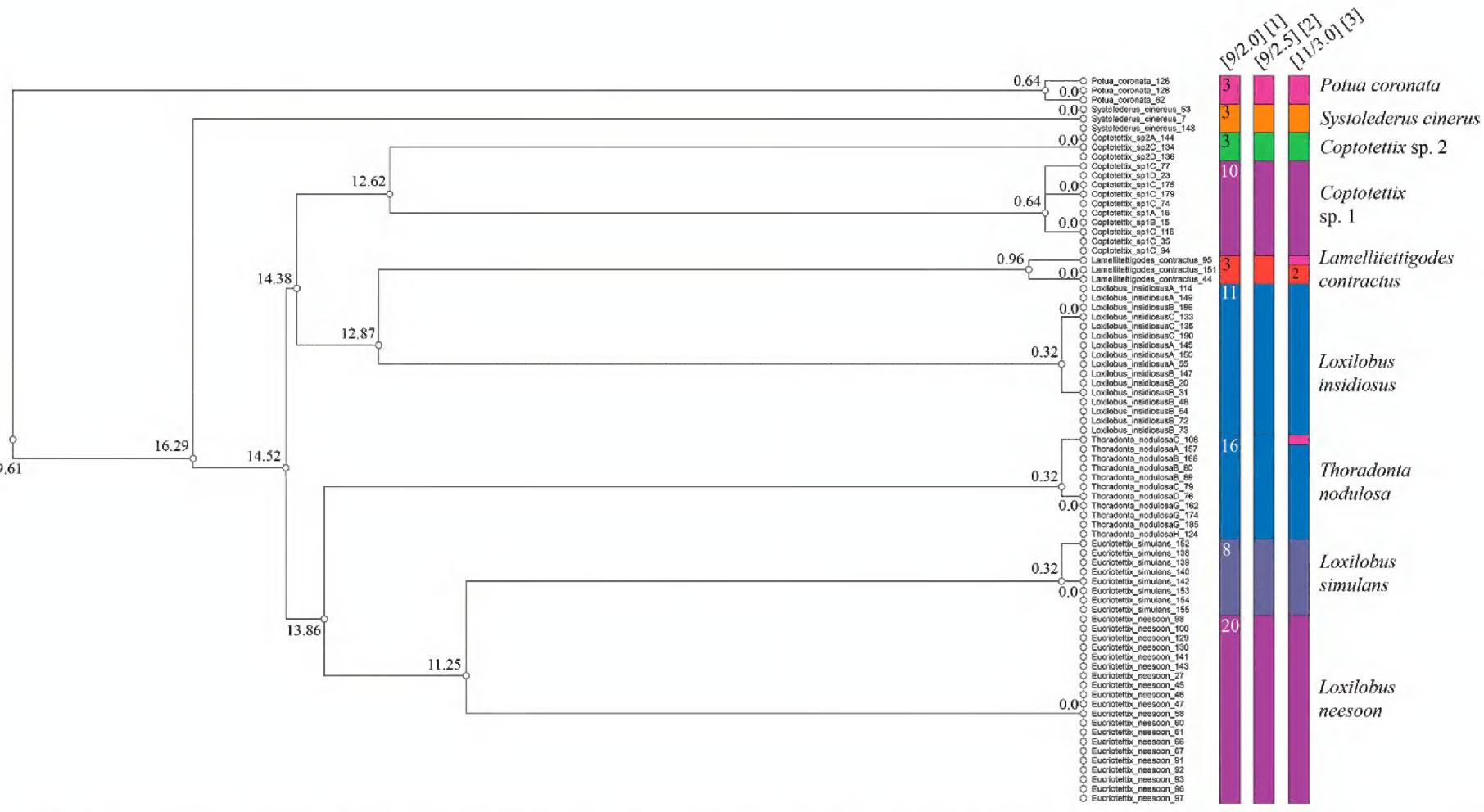

Figure 2. Dendrogram from objective clustering and the first–third best species partitions from ASAP analyses of *COI* sequences of 76 specimens belonging to nine putative species. The values on the branches of the dendrogram represent the genetic distance. For ASAP, the header [x, y] represents the number of species partitions and the asap score; and the number in each species partition represents the number of specimens grouped within the delimited species.

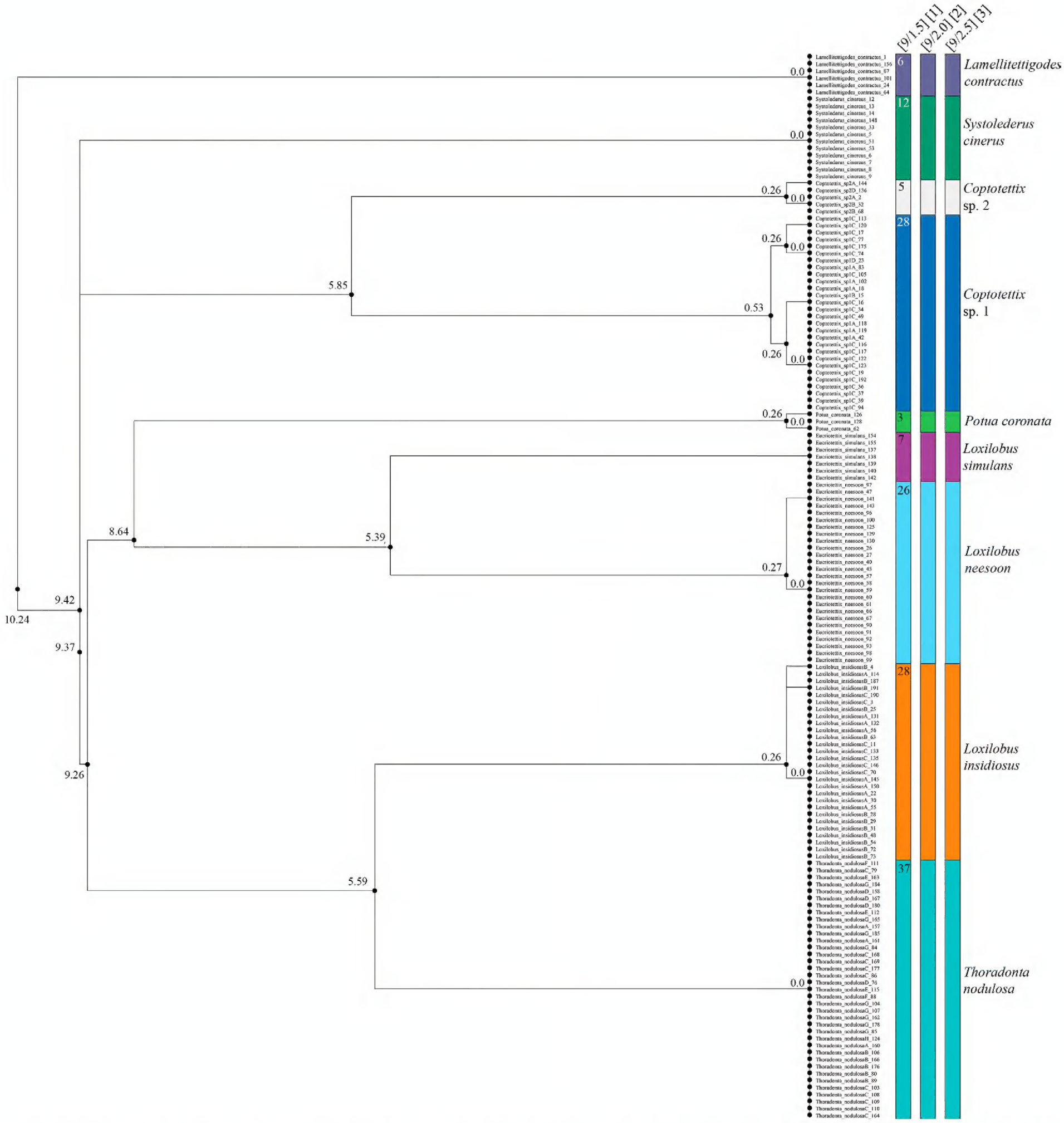

Figure 3. Dendrogram from objective clustering and the first–third best species partitions from ASAP analyses of *12S* sequences of 152 specimens belonging to nine putative species. The values on the branches of the dendrogram represent the genetic distance. For ASAP, the header [x, y] represents the number of species delimited and the asap score; and the number in each partition represents the number of specimens grouped within the delimited species.

nine putative species, the lowest genetic distances range from 5.38–10.24%, once again demonstrating a clear gap in interspecific genetic distance. *Coptotettix* morphospecies 1 and *Coptotettix* morphospecies 2 were genetically very different: between them, specimens had p-distance of at least 5.85%. On the other hand, specimens within morphospecies 1 were split into two genetic clusters, but there was at least one pair of specimens with as low as 0.53% p-distance between these clusters. Similarly, for each of *Coptotettix* morphospecies 2 and *Loxilobus insidiosus*, the last specimen with the highest genetic distance from all other specimens had

at least 0.26% p-distance from one other specimen in their respective clusters. For *Thoradonta nodulosa*, all the specimens in our dataset had the same haplotype. After a global-alignment, the applied ASAP procedure identified nine mOTUs (hypothetical species) at the threshold distance 4.10% which has the best asap-score (1.5). The ASAP results showed that the first- (lowest asap score), second- (next lowest asap score) and third-best partitions show identical results, and corroborates the mOTUs delimitation from objective clustering analysis.

Morphometric analysis

As a proof-of-concept using the two *Loxilobus* cryptic species which do not exhibit color polymorphism, we performed a PCA based on 20 characters (ICL and ICW are not available for the genus) from 27 *Loxilobus neesoon* (Tan & Storozhenko, 2018) and nine *Loxilobus simulans* (Tan & Storozhenko, 2017) specimens. The biplot yields the first two components explaining 76.4% of variance, and the two species show two distinct clusters (Fig. 4A).

Following this, we performed a similar PCA for *Coptotettix* morphospecies 1 and *Coptotettix* morphospecies 2 based on 20 characters from 33 morphospecies 1 and seven morphospecies 2 specimens, which accounts for 70.1% of variance. ICL and ICW were not available for all specimens. The two morphospecies form two distinct clusters, but the color morphs within each morphospecies do not (Fig. 4B). This is most prominent among A, C and D color morphs of morphospecies 1. There was only one specimen for B color morph of morphospecies 1 which did not appear to cluster with the other color morphs. For morphospecies 2, owing to the limited number of specimens, it is not so obvious that the four color morphs overlap in their morphometry. However, it is notable that variations among the color morphs within each morphospecies are smaller than between both morphospecies.

For *Loxilobus insidiosus*, the PCA based on all 22 characters from 36 specimens, which accounts for 58.9% of variance, shows that the three color morphs were indistinguishable (Fig. 4C). Likewise, for *Thoradonta nodulosa*, the PCA based on all 22 characters from 50 specimens, which accounts for 67.3% of variance shows that the eight color morphs were morphometrically similar (Fig. 4D).

Phylogenetic analysis

ML analysis for the *COI* sequences was done using the best-fit model according to BIC, i.e., Transitional Model of Molecular Evolution (TIM2)+F+I+Gamma distribution (G4) (Fig. 5). Most species were recovered as monophyletic groups (all with at least BS of 90%). The only exceptions are the *Coptotettix* comprising of *Coptotettix beihaiensis* Deng & Zheng, 2006, *Coptotettix indicus* Hancock, 1912, *Coptotettix longjiangensis* Zheng & Wei, 2000, and *Coptotettix transimaculatus* Zheng & Jiang, 2002, as well as *Coptotettix cangshanensis* Zheng, Nie & He, 2005 and *Coptotettix longtanensis* Zheng & Jiang, 2004. Generally, closely related species were recovered as distinct monophyletic groups. These include the two morphologically cryptic *Loxilobus* (i.e., *Loxilobus neesoon* and *Loxilobus simulans*) (BS of 94%) and two species of *Systolederus* (BS of 95%). Deeper relationships were generally poorly supported (BS of ≤ 75%), such as potential sister lineages made up of *Loxilobus insidiosus* and *Thoradonta*.

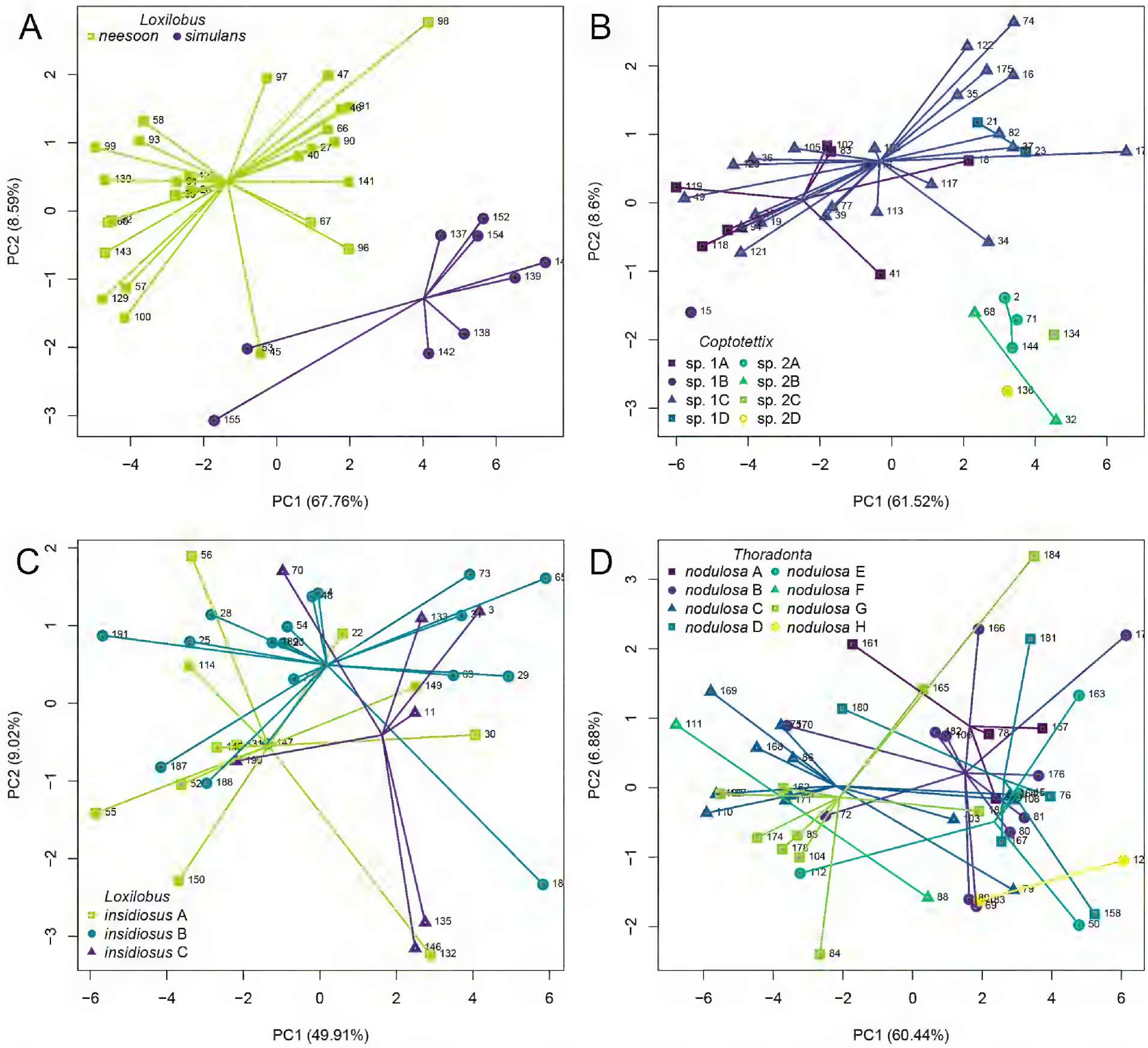

Figure 4. Plots of Principal Component Analysis for morphometry of. **A.** *Loxilobus simulans* and *Loxilobus neesoon*; **B.** *Coptotettix* spp.; **C.** *Loxilobus insidiosus*; **D.** *Thoradonta nodulosa*.

Discussion

Our study on the Tetrigidae from Singapore demonstrates the use of integrative taxonomy to test species-delimitation hypothesis of polymorphic species by incorporating genetic and morphometric data, and can be applied to other tetrigids worldwide. We have also sequenced the first DNA barcode for a small and rare genus, *Potua* (Cladonotinae, Xerophyllini) from Singapore. Species delimitation using *COI* and *12S* loci as well as morphometric analyses of 20 to 22 morphometric characters, unequivocally showed that well-established cryptic species pair *Loxilobus simulans* and *Loxilobus neesoon* can be effectively differentiated. It also demonstrates that color variations observed in *Coptotettix* morphospecies, *Thoradonta nodulosa*, and *Loxilobus insidiosus* do not indicate species differences. Specifically, the color morphs of *Coptotettix* morphospecies 1 (four morphs), *Coptotettix* morphospecies 2 (four morphs), *Thoradonta nodulosa* (three morphs), and *Loxilobus insidiosus* (eight morphs) have very

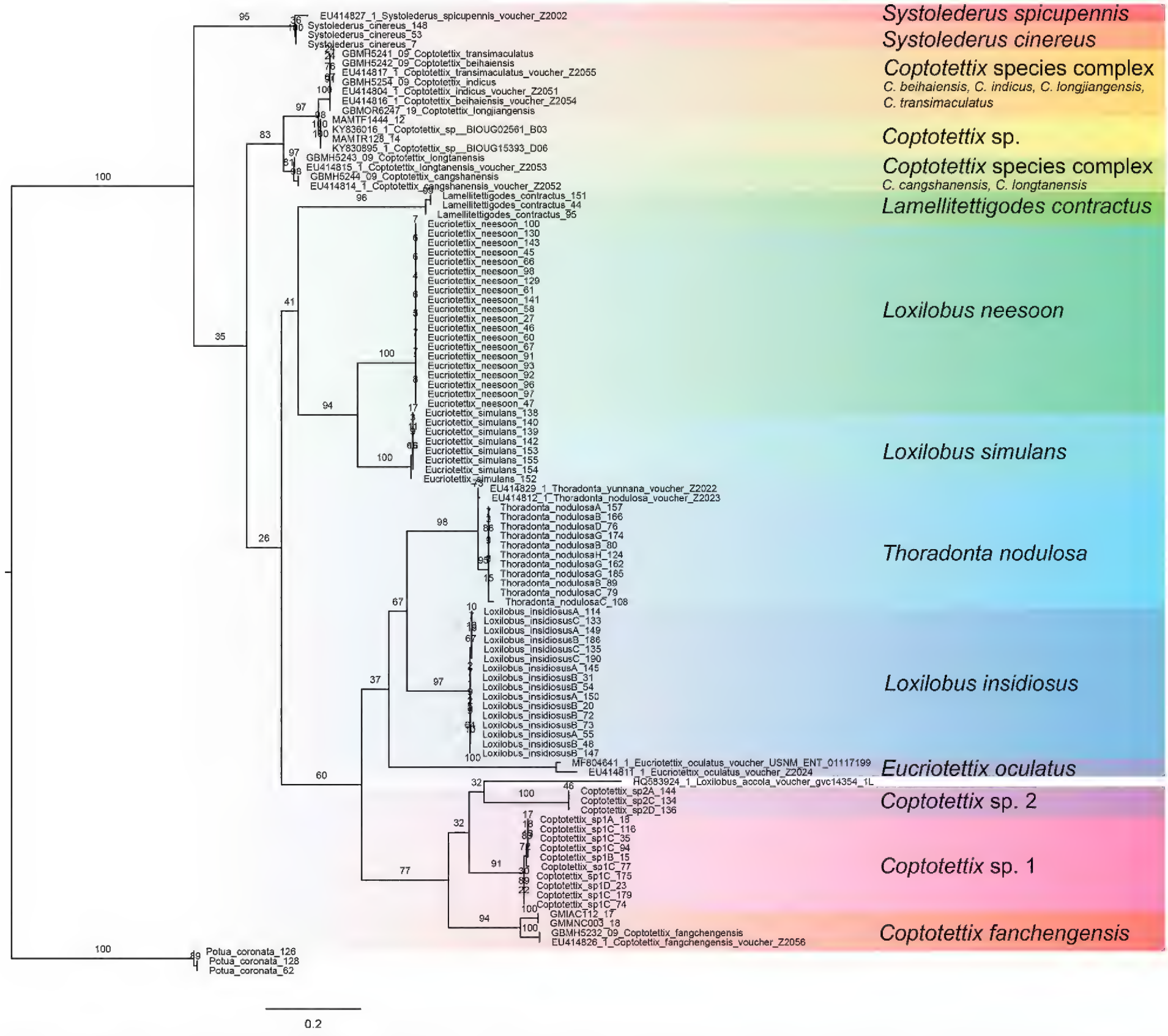

Figure 5. Maximum Likelihood (ML) tree showing the phylogeny of *COI* of Tetrigidae. Support values for ML analyses are given at branches (BS); BS values below 75% are considered as low support.

low intraspecific genetic variability, and are inseparable by morphometry. Intraspecific color variability is widely documented among tetrigids (Holst 1986; Ichikawa et al. 2006; Zhao et al. 2016; Adžić 2021; Long et al. 2023). Within Singapore, for example, color variability has been observed in other tetrigids species, such as *Paratettix variabilis* Bolívar, 1887, *Paratettix curtipennis* (Hancock, 1912) (Tetriginae: Tetrigini), *Ergatettix dorsiferus* (Walker, 1871) (Tetriginae), and *Criotettix robustus* (Hancock, 1907) (Criotettiginae: Criotettigini) (see Tan, 2012). Therefore, in a broader context, our findings can probably apply to other tetrigids exhibiting color morphs, suggesting that any color variations should not be interpreted as species differences first unless otherwise suggested by molecular and/or morphometry evidence.

Intraspecific color polymorphism observed in these pygmy grasshoppers is likely driven by selection pressure, with certain color morphs exhibiting better camouflaging abilities in specific microhabitats (Neo et al. 2023). For instance, Neo et al. (2023) observed that light-colored morphs of *Thoradonta nodulosa* tend to be found among leaf litter and herbaceous cover, whereas dark-colored

morphs are prevalent in exposed microhabitats. This also aligns with the findings of Zha et al. (2016), which showed that body colorations in *Thoradonta* are adapted to their local substrate. However, the genetic basis of the color polymorphism occurring in *Coptotettix* species, *Thoradonta nodulosa*, and *Loxilobus insidiosus* remains unanswered. Further in-depth genomic investigations may be necessary to determine if there is a genetic basis to this color polymorphism.

Incorporating DNA data into future taxonomic work for tetrigids will be important to corroborate species hypothesis from non-reproductive characters, which have been used predominantly to delimit species. At present, the utilization of DNA data for taxonomic studies of tetrigids is very limited. A quick survey of the DNA barcode data available online (data sourced 22 November 2022) found that a great proportion of the data deposited is not identified properly to species or genus levels (Kasalo et al. 2023). The study by Zhao et al. (2016) on *Tetrix bolivari* represents one of the rare instances in which DNA barcoding was used to examine explicitly color polymorphism in pygmy grasshoppers. However, Lehmann et al. (2017) later pointed out that Zhao et al. (2016) had misidentified their study subject, and it should have been a *Paratettix* species. This further reiterates that good alpha taxonomy remains crucial in building reliable DNA barcode databases, which is currently still lacking for tetrigids. Consequently, we also followed the advice of Lehmann et al. (2017), by ensuring that images showing the morphology of the taxa of interest are available (see Neo et al. 2023). We also ensured that the specimens with DNA barcodes are vouchered properly and deposited in an accessible repository for future alpha taxonomic validation and revision, whenever necessary.

The molecular species delimitation using *COI* and *12S* loci can be a reliable tool to identify species, however, it should not be used to infer evolutionary relationships. To provide a very preliminary hypothesis of species relationships, we constructed a phylogenetic tree using predominantly short COI fragments, which generally supported our species delimitation (Fig. 5). We would like to emphasize again that the dendrograms in Figs 2, 3 were generated for both the *COI* and *12S* loci from objective clustering analysis and ASAP and are based on pairwise distances, instead of phylogenetic relationships based on genetic models. Hence, caution should be taken not to use it to make inferences on evolutionary relationships. Preliminary phylogenetic analysis of *COI* sequences (Fig. 5) recovered most species as well-supported monophyletic groups, with the exception of some *Coptotettix* species. Closely related species, including morphologically cryptic taxa, were generally recovered as distinct monophyletic groups. However, the ML tree offers limited insight into deeper evolutionary relationships, as the evolution of one locus does not necessarily represent species evolution and has reduced resolution for deeper nodes (Hajibabaei et al. 2007). Additional biases may arise from the mitochondrial origin of COI owing to incomplete lineage sorting and maternal inheritance (Moritz and Cicero 2004). We recommend that future studies use multiple loci from both mitochondrial and nuclear genomes to make robust phylogenetic inferences (Moritz and Cicero 2004; Hajibabaei et al. 2007; Li et al. 2021, 2025a, 2025b) and to treat our results as preliminary hypotheses.

Morphometric analyses have been shown to offer a more quantitative approach to delimit species of tetrigids, including discovering cryptic species (Tan et al. 2017). However, care should be taken to properly differentiate between intraspecific and interspecific differences. For example, some species,

including *Loxilobus insidiosus* and *Thoradonta nodulosa* exhibit huge intraspecific variation in their pronotum sizes and hind wing lengths. Individuals with different pronotum and hind wing lengths can and have misled taxonomists to erroneously consider them as distinct species (Long et al. 2023). We also observed that deformities in the pronotum are not uncommon, and this can sometimes be natural or an artefact of specimen preservation (Long et al. 2023). It is therefore crucial that these abnormalities should not be used for the analysis to avoid biases or misinterpretation.

Conclusions

Our study highlights the utility and importance of integrative taxonomy for tetrigids. Using alpha taxonomy, we identified color variants observed in four putative species—*Coptotettix* morphospecies 1, *Coptotettix* morphospecies 2, *Loxilobus insidiosus*, and *Thoradonta nodulosa*—collected from a single site in Singapore. Using objective clustering and species delimitation of DNA barcodes, as well as morphometric analysis, we established that discontinuous color variations observed are merely intraspecific variations (color polymorphism) rather than representing different species. This approach can also be used to resolve taxonomic problems in tetrigids.

Acknowledgements

The first author would like to thank Ting Wai Kit, Samuel Lee Ming De, Goh Poh Yee, Teo Jing Xiang Keane, and the NUS Freshwater and Invasion Biology Lab for their field assistance, input, and moral support. The authors thank Josip Skejo and Karmela Adžić (both from the University of Zagreb, Faculty of Science, Department of Biology) for verifying some species identifications. Permission to conduct faunistic surveys and collection of material was granted by the National Parks Board (NP/RP22-096 and NP/RP22-113). The work of the first author was part of NUS Undergraduate Research Opportunities Program in Science (UROPS) and the Research Experience Programme (REx) funded by the Department of Biological Sciences, National University of Singapore. This research did not receive any specific grant from funding agencies in the public, commercial, or not-for-profit sectors. The authors declare no competing interests.

Additional information

Conflict of interest

The authors have declared that no competing interests exist.

Ethical statement

No ethical statement was reported.

Use of AI

No use of AI was reported.

Funding

No funding was reported.

Author contributions

Ivan Neo, Ming Kai Tan, and Darren C. J. Yeo conceptualized the study. Ivan Neo and Rebecca Loh collected the data. Ivan Neo, Rebecca Loh, and Ming Kai Tan analyzed the data and wrote the original draft. Tricia J.Y. Cho, Darren C. J. Yeo, and Nalini Puniamoorthy reviewed and edited the manuscript. Ming Kai Tan, Darren C. J. Yeo, Tricia J.Y. Cho, and Rebecca Loh offered supervision and mentorship.

Author ORCIDs

Ivan Neo https://orcid.org/0009-0006-8729-3847
Rebecca Ker Loh https://orcid.org/0000-0001-9522-6843
Tricia J. Y. Cho https://orcid.org/0009-0004-5332-8154
Nalini Puniamoorthy https://orcid.org/0000-0003-0651-8356
Darren C. J. Yeo https://orcid.org/0000-0003-2669-4349
Ming Kai Tan https://orcid.org/0000-0002-4324-6305

Data availability

All of the data that support the findings of this study are available in the main text or Supplementary Information.

References

Adžić K (2021) Pygmy Grasshoppers (Orthoptera: Tetrigidae) of Peninsular Malaysia. Master's Thesis. University of Zagreb, Zagrebu. https://doi.org/10.1016/B978-0-12-821139-7.00046-5

Ahnesjö J, Forsman A (2003) Correlated evolution of color pattern and body size in polymorphic pygmy grasshoppers, *Tetrix undulata*. Journal of Evolutionary Biology 16(6): 1308–1318. https://doi.org/10.1046/j.1420-9101.2003.00610.x

Beukema W, Nicieza AG, Lourenço A, Velo-Antón G (2016) Color polymorphism in *Salamandra salamandra* (Amphibia: Urodela), revealed by a lack of genetic and environmental differentiation between distinct phenotypes. Journal of Zoological Systematics and Evolutionary Research 54(2): 127–136. https://doi.org/10.1111/jzs.12119

Blow R, Willink B, Svensson EI (2021) A molecular phylogeny of forktail damselflies (genus *Ischnura*) reveals a dynamic macroevolutionary history of female color polymorphisms. Molecular Phylogenetics and Evolution 160: 107134. https://doi.org/10.1016/j.ympev.2021.107134

Bolívar IYU (1887) Essai sur les Acridiens de la tribu des Tettigidae. Annales de la Société entomologique de Belgique 31: 175–313.

Camacho C, Coulouris G, Avagyan V, Ma N, Papadopoulos J, Bealer K, Madden TL (2009) BLAST+: Architecture and applications. BMC Bioinformatics 10(1): 421. https://doi.org/10.1186/1471-2105-10-421

Chamorro-Rengifo J, Lopes-Andrade C (2014) The phallus in Tettigoniidae (Insecta: Orthoptera: Ensifera): revision of morphology and terminology, and discussion on its taxonomic importance and evolution. Zootaxa 3815(2): 151–199. https://doi.org/10.11646/zootaxa.3815.2.1

Chintauan-Marquier IC, Legendre F, Hugel S, Robillard T, Grandcolas P, Nel A, Zuccon D, Desutter-Grandcolas L (2016) Laying the foundations of evolutionary and systematic studies in crickets (Insecta, Orthoptera): A multilocus phylogenetic analysis. Cladistics : The International Journal of the Willi Hennig Society 32(1): 54–81. https://doi.org/10.1111/cla.12114

Cueva Del Castillo R, González-Zertuche M, Ramírez-Delgado VH (2021) Interpopulation variation in sexual dichromatism in the Neotropical grasshopper *Sphenarium purpurascens* (Orthoptera: Pyrgomorphidae). Biological Journal of the Linnean Society. Linnean Society of London 132(4): 900–911. https://doi.org/10.1093/biolinnean/blaa227

Devriese H (1996) Bijdrage tot de systematiek, morfologie en biologie van de West-Palearktische Tetrigidae. Nieuwsbrief Saltabel 15(1): 2–38.

Drosopoulos S (2003) New data on the nature and origin of color polymorphism in the spittlebug genus *Philaenus* (Hemiptera: Aphrophoridae). Annales de la Société Entomologique de France 39(1): 31–42. https://doi.org/10.1080/00379271.2003.10697360

Fianco M (2023) Katydids (Orthoptera: Tettigoniidae) from Guartelá State Park, State of Paraná, Southern Brazil: diversity, bioacoustics and description of five new species. Journal of Natural History 57(17–20): 1080–1137. https://doi.org/10.1080/00222933.2023.2231579

Fite T, Devriese H, Kulanek D, Skejo J, Kasalo N, Misganaw M, Teferea T, Villinger J, Husemann M (2025) Tetrigidae of Ethiopia: First Species Delimitation via DNA Barcoding and Description of Three New Species. Taxonomy 5(3): 49. https://doi.org/10.3390/taxonomy5030049

Forsman A (1997) Thermal capacity of different color morphs on the pygmy grasshopper *Tetrix subulata*. Annales Zoologici Fennici 34: 145–149.

Forsman A (2000) Some like it hot: Intra-population variation in behavioural thermoregulation in color-polymorphic pygmy grasshoppers. Evolutionary Ecology 14(1): 25–38. https://doi.org/10.1023/A:1011024320725

Forsman A, Appelqvist S (1998) Visual predators impose correlational selection on prey color pattern and behavior. Behavioral Ecology 9(4): 409–413. https://doi.org/10.1093/beheco/9.4.409

Geller J, Meyer C, Parker M, Hawk H (2013) Redesign of PCR primers for mitochondrial cytochrome c oxidase subunit I for marine invertebrates and application in all-taxa biotic surveys. Molecular Ecology Resources 13(5): 851–861. https://doi.org/10.1111/1755-0998.12138

Gorochov AV (2015) Evolution and taxonomic significance of the copulatory apparatus in Ensifera (Orthoptera). Part 2: Male genitalia in Grylloidea. Zoosystematica Rossica 24(1): 31–41. https://doi.org/10.31610/zsr/2015.24.1.31

Günther K (1939) Revision der Acrydiinae (Orthoptera), III. Sectio Amorphopi (Metrodorae Bol. 1887, aut.). Abhandlungen und Berichte aus den Staatlichen Museen für Tierkunde und Völkerkunde in Dresden, Series A (Zoology), Neue Folge, 20(Bd. 1), 16–335.

Hajibabaei M, Singer DA, Hebert PD, Hickey DA (2007) DNA barcoding: How it complements taxonomy, molecular phylogenetics and population genetics. Trends in Genetics : TIG 23(4): 167–172. https://doi.org/10.1016/j.tig.2007.02.001

Hancock JL (1907) Studies of the Tetriginae (Orthoptera) in the Oxford University Museum. Transactions of the Entomological Society of London 1907: 213–244. [pl. 21] https://doi.org/10.1111/j.1365-2311.1907.tb01760.x

Hancock JL (1909) Further studies of the Tetriginae (Orthoptera) in the Oxford University Museum. Transactions of the Royal Entomological Society of London 1908(3-4): 387–426. https://doi.org/10.1111/j.1365-2311.1909.tb02160.x

Holst KT (1986) The Saltatoria of northern Europe. Fauna Entomologica Scandinavica 16: 1–127.

Honek A, Furlan L (1995) Color polymorphism in *Agriotes ustulatus* (Coleoptera: Elateridae): Absence of geographic and temporal variation. European Journal of Entomology 92: 437–437.

Hugall AF, Stuart-Fox D (2012) Accelerated speciation in color-polymorphic birds. Nature 485(7400): 631–634. https://doi.org/10.1038/nature11050

Huxley JS (1955) Morphism in birds. In: Portmann A, Sutter E (Eds) Proceedings of the 11th International Ornithological Congress. Birkhäuser, Basel, 309–328.

Ichikawa A, Kano Y, Kawai M, Tominago O, Murai T (2006) Orthoptera of the Japanese Archipelago in Color. Hokkaido University Press, Hokkaido, 688 pp.

Kambhampati S (1995) A phylogeny of cockroaches and related insects based on DNA sequence of mitochondrial ribosomal RNA genes. Proceedings of the National Academy of Sciences of the United States of America 92(6): 2017–2020. https://doi.org/10.1073/pnas.92.6.2017

Kaori T, Atsushi H, Takayoshi N (2010) Camouflage effects of various color-marking morphs against different microhabitat backgrounds in a polymorphic pygmy grasshopper *Tetrix japonica*. PLoS ONE 5(7): 1–7. https://doi.org/10.1371/journal.pone.0011446

Kasalo N, Skejo J, Husemann M (2023) DNA barcoding of pygmy hoppers—The first comprehensive overview of the BOLD Systems' data shows promise for species identification. Diversity 15(6): 696. https://doi.org/10.3390/d15060696

Kasalo N, Janzen DH, Hallwachs W, Brown A, Husemann M, Vielsäcker M, Domazet-Lošo T, Franjevic D, Subedi M, Bogić D, Skejo J (2025) Integrative taxonomy of Costa Rican Tetrigidae (Orthoptera) reveals eight new species. Diversity 17(3): 190. https://doi.org/10.3390/d17030190

Lehmann AW, Devriese H, Tumbrinck J, Skejo J, Lehmann GU, Hochkirch A (2017) The importance of validated alpha taxonomy for phylogenetic and DNA barcoding studies: A comment on species identification of pygmy grasshoppers (Orthoptera, Tetrigidae). ZooKeys 679: 139–144. https://doi.org/10.3897/zookeys.679.12507

Leray M, Yang JY, Meyer CP, Mills SC, Agudelo N, Ranwez V, Boehm JT, Machida RJ (2013) A new versatile primer set targeting a short fragment of the mitochondrial *COI* region for metabarcoding metazoan diversity: Application for characterizing coral reef fish gut contents. Frontiers in Zoology 10(1): 34. https://doi.org/10.1186/1742-9994-10-34

Li R, Ying X, Deng W, Rong W, Li X (2021) Mitochondrial genomes of eight Scelimeninae species (Orthoptera) and their phylogenetic implications within Tetrigoidea. PeerJ 9: e10523. https://doi.org/10.7717/peerj.10523

Li X, Dou W, Lin L (2025a) Mitogenomic phylogeny of Tetrigoidea (Insecta, Orthoptera), with a focus on the genus *Zhengitettix*. PeerJ 13: e19521. https://doi.org/10.7717/peerj.19521

Li X, Dong X, Lin L (2025b) New insights into the phylogeny of Tetrigoidea (Insecta, Orthoptera), with the announcement of the first mitogenome of the genus *Phaesticus*. ZooKeys 1251: 115–130. https://doi.org/10.3897/zookeys.1251.154178

Long Y, Teng C, Huang C, Zhang R, Deng W, Lin L (2023) Twenty-three new synonyms of the Eastern common groundhopper, *Tetrix japonica* (Bolívar, 1887) (Orthoptera, Tetrigidae). ZooKeys 1187: 135–167. https://doi.org/10.3897/zookeys.1187.110067

McKinnon JS, Pierotti ME (2010) Colour polymorphism and correlated characters: Genetic mechanisms and evolution. Molecular Ecology 19(23): 5101–5125. https://doi.org/10.1111/j.1365-294X.2010.04846.x

Meier R, Shiyang K, Vaidya G, Ng PKL (2006) DNA barcoding and taxonomy in Diptera: A tale of high intraspecific variability and low identification success. Systematic Biology 55(5): 715–728. https://doi.org/10.1080/10635150600969864

Meier R, Wong W, Srivathsan A, Foo M (2016) $1 DNA barcodes for reconstructing complex phenomes and finding rare species in specimen-rich samples. Cladistics: The International Journal of the Willi Hennig Society 32(1): 100–110. https://doi.org/10.1111/cla.12115

Messmer V, van Herwerden L, Munday PL, Jones GP (2005) Phylogeography of color polymorphism in the coral reef fish *Pseudochromis fuscus*, from Papua New Guinea and the Great Barrier Reef. Coral Reefs 24(3): 392–402. https://doi.org/10.1007/s00338-005-0001-9

Moritz C, Cicero C (2004) DNA barcoding: Promise and pitfalls. PLoS Biology 2(10): e354. https://doi.org/10.1371/journal.pbio.0020354

Moser V, Baur H, Lehmann AW, Lehmann GUC (2021) Two species?—Limits of the species concepts in the pygmy grasshoppers of the *Tetrix bipunctata* complex (Orthoptera, Tetrigidae). ZooKeys 1043: 33–59. https://doi.org/10.3897/zookeys.1043.68316

Muhammad AA, Tan MK, Abdullah NA, Azirun MS, Bhaskar D, Skejo J (2018) An annotated catalogue of the pygmy grasshoppers of the tribe Scelimenini Bolívar, 1887 (Orthoptera: Tetrigidae) with two new *Scelimena* species from the Malay Peninsula and Sumatra. Zootaxa 4485(1): 1–70. https://doi.org/10.11646/zootaxa.4485.1.1

Nabours RK (1929) The Genetics of the Tettigidae (Grouse Locusts). Springer Netherlands, Dordrecht, 92 pp. https://doi.org/10.1007/978-94-011-9487-7

Nabours RK, Foster ME (1929) Parthenogenesis and the inheritance of color patterns in the grouse locust *Paratettix texanus* Hancock. The Biological Bulletin 56(2): 129–155. https://doi.org/10.2307/1536922

Neo I, Tan MK, Cho TJY, Yeo DCJ (2023) A faunistic study and taxonomic account of species of pygmy grasshoppers (Orthoptera: Tetrigidae) from Singapore's last freshwater swamp forest. Journal of Asia-Pacific Biodiversity 17(1): 87–116. https://doi.org/10.1016/j.japb.2023.09.006

Nie RE, Xue HJ, Hua Y, Yang XK, Vogler AP (2012) Distinct species or color polymorphism? Life history, morphology and sequence data separate two *Pyrrhalta* elm beetles (Coleoptera: Chrysomelidae). Systematics and Biodiversity 10(2): 133–146. https://doi.org/10.1080/14772000.2012.687783

Pan ZX, Hong F, Jiang GF (2018) Morphometrics reveal correlation between morphology and bioclimatic factors and population mixture in *Tetrix japonica* (Orthoptera: Tetrigidae). Acta Zoologica (Stockholm, Sweden) 99(2): 199–210. https://doi.org/10.1111/azo.12240

Puillandre N, Brouillet S, Achaz G (2021) ASAP: Assemble species by automatic partitioning. Molecular Ecology Resources 21(2): 609–620. https://doi.org/10.1111/1755-0998.13281

Ramaiah M, Meshram NM, Dey D (2023) Integrative approaches establish color polymorphism in the bamboo-feeding leafhopper *Mukaria splendida* Distant (Hemiptera: Cicadellidae) from India. Insects 14(1): 44. https://doi.org/10.3390/insects14010044

Roulin A, Bize P (2007) Sexual selection in genetic color-polymorphic species: A review of experimental studies and perspectives. Journal of Ethology 25(2): 99–105. https://doi.org/10.1007/s10164-006-0006-z

Roulin A, Wink M (2004) Predator-prey relationships and the evolution of color polymorphism: A comparative analysis in diurnal raptors. Biological Journal of the Linnean

Society. Linnean Society of London 81(4): 565–578. https://doi.org/10.1111/j.1095-8312.2004.00308.x

Sánchez-Guillén RA, Hammers M, Hansson B, Van Gossum H, Cordero-Rivera A, Galicia Mendoza DI, Wellenreuther M (2013) Ontogenetic shifts in male mating preference and morph-specific polyandry in a female color polymorphic insect. BMC Evolutionary Biology 13(1): 1–11. https://doi.org/10.1186/1471-2148-13-116

Skejo J, Pushkar TI, Kasalo N, Pavlović M, Deranja M, Adžić K, Tan MK, Rebrina F, Muhammad AA, Abdullah NA, Japir R, Chung AYC, Tumbrinck J (2022) Spiky pygmy devils: revision of the genus *Discotettix* (Orthoptera: Tetrigidae) and synonymy of Discotettiginae with Scelimeninae. Zootaxa 5217(1): 1–64. https://doi.org/10.11646/zootaxa.5217.1.1

Šlenker M, Koutecký P, Marhold K (2022) MorphoTools2: An R package for multivariate morphometric analysis. Bioinformatics (Oxford, England) 38(10): 2954–2955. https://doi.org/10.1093/bioinformatics/btac173

Song H (2010) Grasshopper systematics: Past, present and future. Journal of Orthoptera Research 19(1): 57–68. https://doi.org/10.1665/034.019.0112

Song H, Amédégnato C, Cigliano MM, Desutter-Grandcolas L, Heads SW, Huang Y, Otte D, Whiting MF (2015) 300 million years of diversification: Elucidating the patterns of orthopteran evolution based on comprehensive taxon and gene sampling. Cladistics: The International Journal of the Willi Hennig Society 31(6): 621–651. https://doi.org/10.1111/cla.12116

Srivathsan A, Lee L, Katoh K, Hartop E, Kutty SN, Wong J, Yeo D, Meier R (2021) ONTbarcoder and MinION barcodes aid biodiversity discovery and identification by everyone, for everyone. BMC Biology 19(1): 217. https://doi.org/10.1186/s12915-021-01141-x

Tan MK (2012) Orthoptera in the Bukit Timah and Central Catchment Nature Reserves (Part 1): Suborder Caelifera. Raffles Museum of Biodiversity Research, National University Singapore, Singapore, 40 pp. Uploaded 4 May 2012. https://lkcnhm.nus.edu.sg/wp-content/uploads/sites/11/app/uploads/2017/04/caelifera_btnr_ccnr.pdf

Tan MK, Storozhenko SY, Hwang WS, Meier R (2017) Integrative taxonomy reveals two sympatric species of the genus *Eucriotettix* Hebard, 1930 (Orthoptera: Tetrigidae). Zootaxa 4268(3): 377–394. https://doi.org/10.11646/zootaxa.4268.3.4

Tan MK, Tumbrinck J, Baroga-Barbecho JB, Yap SA (2019) A new species and morphometric analysis of *Cladonotella* (Tetrigidae: Cladonotinae). Journal of Orthoptera Research 28(2): 129–135. https://doi.org/10.3897/jor.28.32464

Trifinopoulos J, Nguyen LT, von Haeseler A, Minh BQ (2016) W-IQ-TREE: A fast online phylogenetic tool for maximum likelihood analysis. Nucleic Acids Research 44(W1): 232–235. https://doi.org/10.1093/nar/gkw256

Tumbrinck J (2014) Taxonomic revision of the Cladonotinae (Orthoptera: Tetrigidae) from the islands of South-East Asia and from Australia, with general remarks to the classification and morphology of the Tetrigidae and descriptions of new genera and species from New Guinea and New Caledonia. In: Telnov D (Ed.), Biodiversity, Biogeography and Nature Conservation in Wallacea and New Guinea II. Entomological Society of Latvia, Riga, 350–396.

Tumbrinck J, Skejo J (2017) Taxonomic and biogeographic revision of the New Guinean genus *Ophiotettix* Walker, 1871 (Tetrigidae: Metrodorinae: Ophiotettigini trib. nov.), with the descriptions of 33 new species. In: Telnov D, Barclay MVL, Pauwels OSG (eds.), Biodiversity, biogeography and nature conservation in Wallacea and New Guinea. Vol. 3. Entomological Society of Latvia, Riga, 525–580.

Wang WY, Srivathsan A, Foo M, Yamane SK, Meier R (2018) Sorting specimen-rich invertebrate samples with cost-effective NGS barcodes: Validating a reverse workflow for specimen processing. Molecular Ecology Resources 18(3): 490–501. https://doi.org/10.1111/1755-0998.12751

Wellenreuther M, Svensson EI, Hansson B (2014) Sexual selection and genetic color polymorphisms in animals. Molecular Ecology 23(22): 5398–5414. https://doi.org/10.1111/mec.12935

Yeo D, Srivathsan A, Meier R (2020) Longer is not always better: Optimizing barcode length for large-scale species discovery and identification. Systematic Biology 69(5): 999–1015. https://doi.org/10.1093/sysbio/syaa014

Zha LS, Sheng M, Wen T, Hyde K (2016) A new species and a revised key of the genus *Thoradonta* (Orthoptera, Tetrigidae). ZooKeys 607: 69–79. https://doi.org/10.3897/zookeys.607.9056

Zha LS, Skejo J, Mao BY, Ding JH (2021) Taxonomic revision of *Phaesticus* Uvarov and synonymy with *Flatocerus* Liang and Zheng syn. nov. (Orthoptera: Tetrigidae). Zootaxa 4965(3): 501–514. https://doi.org/10.11646/zootaxa.4965.3.5

Zhao L, Lin LL, Zheng ZM (2016) DNA barcoding reveals polymorphism in the pygmy grasshopper *Tetrix bolivari* (Orthoptera, Tetrigidae). ZooKeys 582: 111–120. https://doi.org/10.3897/zookeys.582.6301

Supplementary material 1

Material examined

Authors: Ivan Neo, Rebecca Ker Loh, Tricia J. Y. Cho, Nalini Puniamoorthy, Darren C. J. Yeo, Ming Kai Tan

Data type: xlsx

Copyright notice: This dataset is made available under the Open Database License (http://opendatacommons.org/licenses/odbl/1.0/). The Open Database License (ODbL) is a license agreement intended to allow users to freely share, modify, and use this Dataset while maintaining this same freedom for others, provided that the original source and author(s) are credited.

Link: https://doi.org/10.3897/zookeys.1257.163020.suppl1

Supplementary material 2

COI sequences

Authors: Ivan Neo, Rebecca Ker Loh, Tricia J. Y. Cho, Nalini Puniamoorthy, Darren C. J. Yeo, Ming Kai Tan

Data type: txt

Copyright notice: This dataset is made available under the Open Database License (http://opendatacommons.org/licenses/odbl/1.0/). The Open Database License (ODbL) is a license agreement intended to allow users to freely share, modify, and use this Dataset while maintaining this same freedom for others, provided that the original source and author(s) are credited.

Link: https://doi.org/10.3897/zookeys.1257.163020.suppl2

Supplementary material 3

12S sequences

Authors: Ivan Neo, Rebecca Ker Loh, Tricia J. Y. Cho, Nalini Puniamoorthy, Darren C. J. Yeo, Ming Kai Tan

Data type: txt

Copyright notice: This dataset is made available under the Open Database License (http://opendatacommons.org/licenses/odbl/1.0/). The Open Database License (ODbL) is a license agreement intended to allow users to freely share, modify, and use this Dataset while maintaining this same freedom for others, provided that the original source and author(s) are credited.

Link: https://doi.org/10.3897/zookeys.1257.163020.suppl3

Supplementary material 4

Pair-wise distance of COI sequences

Authors: Ivan Neo, Rebecca Ker Loh, Tricia J. Y. Cho, Nalini Puniamoorthy, Darren C. J. Yeo, Ming Kai Tan

Data type: csv

Explanation note: Table showing pair-wise distance of COI sequences between specimens studied here.

Copyright notice: This dataset is made available under the Open Database License (http://opendatacommons.org/licenses/odbl/1.0/). The Open Database License (ODbL) is a license agreement intended to allow users to freely share, modify, and use this Dataset while maintaining this same freedom for others, provided that the original source and author(s) are credited.

Link: https://doi.org/10.3897/zookeys.1257.163020.suppl4

Supplementary material 5

Pair-wise distance of 12S sequences

Authors: Ivan Neo, Rebecca Ker Loh, Tricia J. Y. Cho, Nalini Puniamoorthy, Darren C. J. Yeo, Ming Kai Tan

Data type: csv

Explanation note: Table showing pair-wise distance of 12S sequences between specimens studied here.

Copyright notice: This dataset is made available under the Open Database License (http://opendatacommons.org/licenses/odbl/1.0/). The Open Database License (ODbL) is a license agreement intended to allow users to freely share, modify, and use this Dataset while maintaining this same freedom for others, provided that the original source and author(s) are credited.

Link: https://doi.org/10.3897/zookeys.1257.163020.suppl5